In the matter of
German Application No. 103 04 448.5

I, Dr Andreas Blecha

hereby solemnly and sincerely declare that, to the best of my knowledge and belief, the following document is a true and correct translation of German patent application DE 103 04 448.5, filed with the German Patent and Trademark Office 4 February 2003.

Date: 23 December 2009

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**The attached documents are a correct and accurate reproduction of the original submission for this Application.**

Munich, 10 December 2003
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# Fluorimetric determination of analytes by amine-N-oxides as redox indicators

## Description

The invention concerns methods and reagent kits for the fluorimetric determination of analytes.

There are numerous methods for determining analytes for example for diagnostic applications. One method is to determine the analyte by means of a redox reaction and a redox indicator. In this case an oxidizing or reducing system acts directly on the redox indicator or via a mediator. The presence of the analyte leads to a reduction or oxidation of the redox indicator which enables a qualitative or quantitative determination to be carried out.

Depending on the type of redox indicator that is used, the indicator can be determined by a colorimetric, fluorimetric or electrochemical detection method. Examples of colorimetric detection reagents are heteropolyacids (EP-B-0 431 456), tetrazolium compounds (EP-B-0 574 769), nitrosoaromatic compounds (EP-A-0 620 283), RIND compounds (EP-B-0 190 740), phenazines (WO 93/06487) and indanthrones (EP-B-0 831 327). Examples of electrochemical detection reagents are nitrosoaromatics, phenazines, potassium hexacyanoferrate and benzoquinones (cf. e.g. EP-A-0 441 222 and EP-A-0 505 494). Examples of fluorimetric detection reagents are e.g. resazurin (US 5,912,139), transition metal complexes (Ryabov et al., JBIC 4 (1999) 175-182; Woltman et al., Anal. Chem. 71 (1999) 1504-1512) and scopoletin, esculetin, p-hydroxyphenylacetic acid, di-chlorofluorescein, N-acetyl-3,7-dihydroxyphenoxazine and MNBDH which are used exclusively for the detection of $H_2O_2$ (see also R. Haughland, Handbook of Fluorescent Probes and Research Chemicals, 6$^{th}$ edition 1996).

However, the fluorimetric detection reagents known from the prior art have some disadvantages. Thus most known fluorescent indicators require that metabolites such as glucose are determined by detecting $H_2O_2$ generated by glucose oxidase. This

reaction usually has to be catalytically supported by the enzyme peroxidase and is very prone to interference by electron donors such as urea or bilirubin. The reagents are also not stable for long time periods.

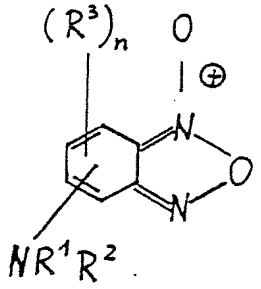
In contrast, redox indicators that allow an oxygen-independent detection of glucose i.e. which directly accept an electron from an oxidizing enzyme instead of oxygen, are advantageous. However, only resazurin and Os and Ru complexes are known to be suitable electron acceptors for this. However, in the case of resazurin the emission bands of the resorufin formed by the redox reaction strongly overlap the absorption bands of non-reacted resazurin which considerably reduces the sensitivity of the analyte determination. The high redox potential of transition metal complexes (e.g. Ru complexes) results in a strong interference by compounds such as ascorbic acid. Their fluorescence efficiency also varies with the oxygen content of the sample.

Furthermore in the case of the previously known fluorescent indicators the excitation light sources used are mainly limited to the UV and green range of light. Thus for example an inadequate number of compounds are known which allow use of the particularly strong blue and red LEDs.

Hence one object of the present invention was to provide new redox-active compounds as detection reagents for the fluorimetric determination of analytes which enable the disadvantages of the prior art to be at least partially eliminated.

This object is achieved according to the invention by providing the N-oxide of NBD-amine or derivatives thereof as redox indicators. The NBD-amine formed by reduction is characterized by a high fluorescence and can be very readily excited with blue light radiation.

Hence a first aspect of the present invention is a method for detecting an analyte by a redox reaction and a fluorimetric determination characterized in that a sample containing the analyte is contacted with a detection reagent which contains a compound of the general formula (I) as a fluorimetric redox indicator:



(I)

in which $R^1$ and $R^2$ are each independently selected from R, $(CH_2CH_2O)_mR$, COR, COOR and OCOR,

$R^3$ in each case is independently selected from $NO_2$, CN, R, OR, OCOR, COOR, SR and halogen,

R is H or $C_1$-$C_4$ alkyl, where alkyl is optionally substituted with halogen, OR, SR, $NR_2$, COOR, $CONR_2$, $SO_3R$ and salts thereof or/and $PO(OR)_3$ and salts thereof, m is an integer from 1-20, preferably from 1-10 and n is 1, 2 or 3.

Another aspect of the invention is a reagent for detecting an analyte by a redox reaction and a fluorimetric determination which contains a compound of the general formula (I) as described above as the fluorimetric redox indicator.

The present invention is suitable for detecting any analytes that can be determined by a redox reaction. The detection can be qualitative, semi-quantitative or quantitative. In one embodiment of the invention the analyte can be a reducible or oxidizable substance, for example a metabolite present in a body fluid such as blood, serum, plasma, urine etc. In this case it is expedient to use a detection reagent which, in addition to the redox indicator, also contains one or more enzymes for reducing or oxidizing the analyte and optionally coenzymes such as nicotine nucleoside derivatives e.g. $NAD^+$, $NADP^+$ or flavin nucleoside derivatives e.g. FAD. Preferred examples of such analytes are glucose, lactate, alcohol, galactose, cholesterol, fructose, glycerol, pyruvate, creatinine, alanine, phenylalanine, leucine, triglycerides, HDL-cholesterol. Glucose can for example be detected by known methods using

glucose oxidase (GOD), glucose dye oxidoreductase (GlucDOR) or glucose dehydrogenase (GDH)/diaphorase.

Furthermore the analyte may also be an enzyme that catalyses a redox reaction, for example an oxidoreductase such as glucose oxidase, glucose dye oxidoreductase, dehydrogenases or an enzyme whose reaction can be coupled to an oxidoreductase reaction.

In addition to the redox indicator and, if required, an enzyme for reducing or oxidizing the analyte, the detection reagent can additionally contain common components such as coenzymes, auxiliary substances, buffers and optionally mediators. Substances are suitable as mediators which support the acceptance of electrons by the redox indicator (I). However, in general those redox indicators are preferred which can directly accept electrons.

The method according to the invention is carried out in conventional test formats such as in dry or wet tests. In a dry test an absorbent material, e.g. in the form of a test strip, is used as a support on which the detection reagent can be applied in a dry form e.g. as a lyophilisate. Liquid tests are carried out in a liquid phase in suitable reaction vessels e.g. cuvettes, microtitre plates etc. where the detection reagent can be provided in the reaction vessel itself or in separate containers in a dry or liquid form.

For a fluorimetric determination, the sample is irradiated with excitation light of a predetermined wavelength and the fluorescence emission light emitted by the sample that has a different wavelength is determined by known methods. Suitable variation of the substituents $R^1$, $R^2$ and $R^3$ enables the present invention to provide optimized test formats for the determination of any analytes.

It is preferable that the redox indicator (I) has one or more hydrophilic groups, e.g. OH groups, COOH groups etc., to increase solubility. In a particularly preferred embodiment $R^1$ and $R^2$ are $C_{1-2}$ alkyl groups substituted with OH such as

hydroxyethyl groups or polyoxyethylene groups. $R^3$ is preferably $NO_2$ and n is 1. A particularly preferred example of a redox indicator according to the invention is shown in figure 1.

Another aspect of the invention is a reagent for detecting an analyte by a redox reaction and a fluorimetric determination comprising a compound of the general formula (I) as stated above as the redox indicator.

In addition to the redox indicator, the reagent according to the invention can also contain other components selected from enzymes, coenzymes, auxiliary substances, buffers and mediators.

The present invention is further elucidated by the following figures and the example.

**Figure 1** shows the N-oxide of the NBD-amine as an example of a redox indicator according to the invention.

**Figure 2** shows the kinetics of the NBD-amine-N-oxide reduction in a system for detecting glucose at various glucose concentrations.

**Example: Glucose determination using an NBD-amine-N-oxide as the redox indicator**

The following compounds were added to a 3 ml fluorescence cuvette (the stated concentrations refer to the final concentration in the cuvette; the N-oxide of the NBD-amine was prepared according to P.B. Ghosh, M.W. Whitehouse, J. Med. Chem., 11, 305-311 (1968)):

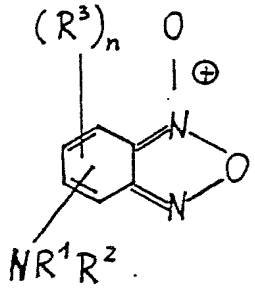
| | |
|---|---|
| glucose dehydrogenase (GlucDH): | 1.3 U/ml |
| diaphorase: | 1.3 U/ml |
| $NAD^+$: | 36.9 µmol/l |
| N-oxide of the NBD-amine: | 35.4 µmol/l |

The reaction was started by adding an aqueous glucose solution (0.1 M phosphate buffer, pH 7.4 containing 1 % NaCl). The kinetics of the reaction were recorded for various glucose concentrations at an excitation wavelength of 470 nm and an emission wavelength of 560 nm. The result of the experiment is shown in figure 2.

Figure 2, in which the intensity of the fluorescence signal (intensity) in impulses per second (cps) is plotted versus time in seconds (sec), shows that an increase in fluorescence is found which is proportional to the glucose concentration present in the sample. In this case the measuring curves 1 to 5 correspond to glucose concentrations of 0, 0.06, 1.2, 2.4 and 4 (each in mg/dl).

## Claims

1. Method for detecting an analyte by a redox reaction and a fluorimetric determination,
**characterized in that**
a sample containing the analyte is contacted with a detection reagent which contains a compound of the general formula (I) as a fluorimetric redox indicator:



(I)

in which $R^1$ and $R^2$ are each independently selected from R, $(CH_2CH_2O)_mR$, COR, COOR and OCOR,
$R^3$ in each case is independently selected from $NO_2$, CN, R, OR, OCOR, COOR, SR and halogen,
R is H or $C_1$-$C_4$ alkyl, where alkyl is optionally substituted with halogen, OR, SR, $NR_2$, COOR, $CONR_2$, $SO_3R$ and salts thereof or/and $PO(OR)_3$ and salts thereof,
m is an integer from 1-20 and
n is 1, 2 or 3.

2. Method as claimed in claim 1,
**characterized in that**
$R^1$ and $R^2$ are a $C_1$-$C_2$ alkyl group substituted with OH.

3. Method as claimed in claim 1 or 2,
**characterized in that**
$R^3$ is $NO_2$.

4. Method as claimed in one of the claims 1 to 3,
**characterized in that**
the redox indicator (I) can directly accept electrons.

5. Method as claimed in one of the claims 1 to 3,
**characterized in that**
the redox indicator (I) can accept electrons via a mediator.

6. Method as claimed in one of the claims 1 to 5,
**characterized in that**
an oxidizable substance is detected as the analyte.

7. Method as claimed in claim 6,
**characterized in that**
a detection reagent is used which additionally contains one or more enzymes for reducing or oxidizing the analyte and optionally a coenzyme.

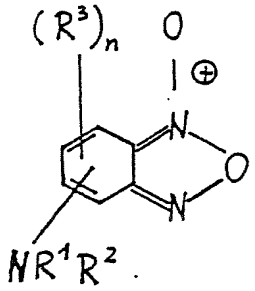
8. Method as claimed in claim 6 or 7,
**characterized in that**
glucose, lactate, alcohol, galactose, cholesterol, fructose, glycerol, pyruvate, creatinine, alanine, phenylalanine, leucine, triglycerides or HDL cholesterol are detected as analytes.

9. Method as claimed in claim 8,
**characterized in that**
glucose is detected using glucose oxidase, glucose dye oxidoreductase or glucose dehydrogenase/diaphorase.

10. Method as claimed in one of the claims 1 to 5,
**characterized in that**
an enzyme catalysing a redox reaction or an enzyme whose reaction can be coupled to an oxidoreductase reaction is detected as the analyte.

11. Method as claimed in claim 10,
**characterized in that**
glutamate-oxalacetate transaminase (GOT), (AST), glutamate-pyruvate transaminase (GPT), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) or creatine kinase (CK) are detected as analytes.

12. Reagent for detecting an analyte by a redox reaction and a fluorimetric determination comprising a compound of the general formula (I):



(I)

in which $R^1$ and $R^2$ are each independently selected from R, $(CH_2CH_2O)_mR$, COR, COOR and OCOR,
$R^3$ in each case is independently selected from $NO_2$, CN, R, OR, OCOR, COOR, SR and halogen,
R is H or $C_1$-$C_4$ alkyl, where alkyl is optionally substituted with halogen, OR, SR, $NR_2$, COOR, $CONR_2$, $SO_3R$ and salts thereof or/and $PO(OR)_3$ and salts thereof,
m is an integer from 1-20 and
n is 1, 2 or 3.

13. Reagent as claimed in claim 12, comprising further components selected from enzymes, coenzymes, auxiliary substances, buffers and mediators.

## Abstract

The invention concerns methods and reagent kits for the fluorimetric determination of analytes.

Fig. 1

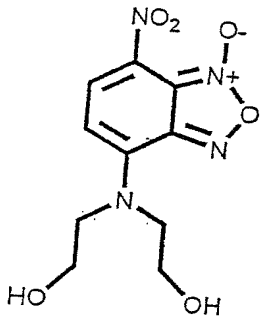
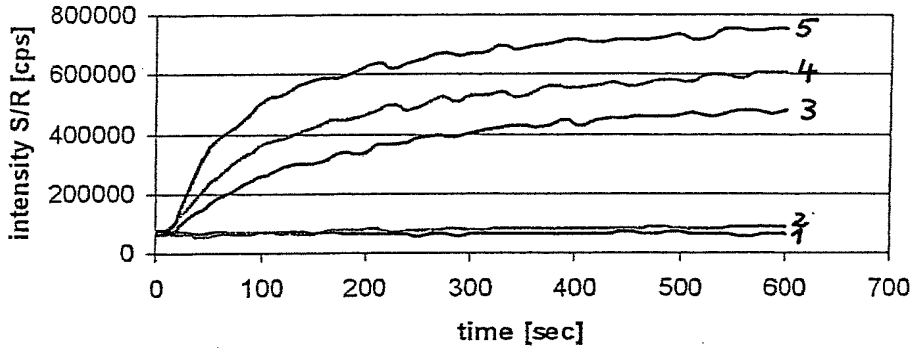

NO2
O-
N+
O
N
N
HO
OH

Fig. 2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300
400
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600
700
time [sec]
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4
3
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